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Mycorrhizal Inoculation Increases Growth and Induces Changes in Specific Polyphenol Levels in Olive Saplings

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Abstract

This study was conducted to investigate the effect of mycorrhizal symbiosis on the levels of polyphenols in olive saplings. Rooted stem cuttings of olive cultivar, 'Arbequina', were inoculated with AM fungus *Rhizophagus intraradices.* The inoculated plants showed more robust growth after six months, and after nine months the increase in the mycorrhizal plant's height was 146%, and the increase in number of leaves was 117% when compared to uninoculated controls. Polyphenols in the methanol extracts of leaves were separated by HPLC and the peaks identified by using commercially available standard compounds and comparing retention time and the mass obtained with the mass spectrometer. Oleuropein, which is a major component of the olive leaf polyphenols, increased in mycorrhizal plants compared to uninoculated plants by 42%, and its derivatives, oleuroside and ligstroside, increased by 68% and 48%, respectively. The highest increase was found in the levels of luteolin-7'-O-glucoside (107% increase), while its sister compound luteolin-4'-O-glucoside increased by 43%. Only verbascoside levels were lower in mycorrhizal plants versus non-mycorrhizal plants declining to below detectable limits. Thus, inoculation of olive saplings with mycorrhizal fungi produces very positive effects on the levels of olive leaf polyphenols. Higher levels polyphenols mean better quality of leaf material for use as herbal medicine.

Keywords: ligstroside, luteolin-7'-O-glucoside, oleuropein, rooting of olive cuttings, verbascoside, oleuroside

1. Introduction

Olive (*Olea europaea*) trees are perhaps the original fruit trees that were cultivated (Zohary & Spiegel, 1975). To many, they are considered holy as these trees have been mentioned in Jewish, Christian and Muslim scriptures (Malik, 2014). However, their present day fame has more to do with the presence of specific health benefitting polyphenols in their fruits and leaves (Soler-Rivas et al., 2000; Tripoli et al., 2005; Uccella, 2001). For example, oleuropein and its derivatives act as antioxidants (Baldioli et al., 1996), and are known to reduce the risks of cancer (Owen et al., 2000; Soler-Rivas et al., 2000; Tripoli et al., 2005) and cardiovascular (Covas, 2007; Manna et al., 2002; Visioli et al., 1998; Wiseman et al., 1996), microbial and even viral diseases (Bisignano et al., 1999; Federici & Bongi, 1983; Fleming et al., 1973; Lee-Huang et al., 2003). Because of such profound benefits of olive polyphenols, consumption of olive oil and other olive products (including leaf extracts) has been steadily increasing and so is the cultivation of olive on new lands (Connor, 2005; Malik & Bradford, 2004; Malik, 2011; Sebestiani et al., 2006). It is therefore logical to devise better methods for production or to be able to grow olives in poor or saline soils. Mycorrhizal symbiosis is considered an important method to aid the cultivation of field crops, including olives, even under high salinity or in areas prone to water or nutrient deficiencies (Artursson et al., 2006; Auge, 2004; Dag et al., 2009; Farzaneh et al., 2011; Menge, 1983; Porras-Soriano et al., 2009).

Mycorrhizal fungi form symbiotic associations with most terrestrial plants (Simon et al., 1993; Smith & Read, 1997). The plant provides photosynthates to the fungi and in turn the hyphae of arbuscular mycorrhizal (AM) fungus extend into the soil and provide water and nutrients, especially the immobile nutrients, to plants (Koide, 1991; Marschner & Dell, 1994). Therefore, increased tolerance to drought (Allen & Boosalis, 1983; Auge, 2004; Nelsen & Safir, 1982; Ruiz-Lozano et al., 1995) and significant improvement in growth and productivity of several plant species have been reported in mycorrhizal plants under water and nutrient deficiencies (Baslam et al., 2011; Citernesi et al., 1998; Estaun et al., 2003; Gerdemann, 1968; Mosse et al., 1975). In addition, protection against pathogens in mycorrhizal plants has also been shown in different cultivars (Castillo et al.,

2006; Espinosa et al., 2014; Liu et al., 2007; Pozo & Azcon-Aguilar, 2007; Rabie, 1998; Watanarojanaporn et al., 2011). Since polyphenols play an important role in plant resistance to pests (Corcuera, 1993; Feeny, 1976; Jones & Klocke, 1987; Lattanzio, 2006; Nicholson & Hammerschmidt, 1992) and are of immense benefit to human health, we started to study the role of mycorrhizae in changing the levels of polyphenols in different plants (Malik et al., 2015a, 2015b). Our initial studies showed that while some polyphenols increased in mycorrhizal plants compared to uninoculated control plants, some other polyphenol species actually decreased (Malik et al., 2015a, 2015b). This study was therefore conducted to investigate whether or not the polyphenols of olive leaves (including the prized polyphenol 'oleuropein') increase in the leaves of mycorrhizal vs non-mycorrhizal olive saplings.

2. Materials and Methods

2.1 Plant Growth and Propagation

Thirty freshly rooted saplings of olive cultivar 'Arbequina' were gifted to us by Mr. Jim Henry, owner of 'Texas Olive Ranch' Carrizo Springs, TX. Samples of roots taken from several saplings indicated that they had established symbiosis with arbuscular mycorrhizal [AM] fungi and therefore, could not be used directly for this study due to lack of uninoculated controls. Therefore, these saplings were grown for 1 year (so that the these plants become big enough so we could take fresh cuttings from these plants to induce new clean roots) in 22x38 cm plastic pots filled with commercial growth media (Premier 'Pro-mix-BX', supplied by Premier Horticulture Inc., Quakertown, PA). The plants were supplied with the commercial nutrient 'Miracle Grow' as per manufacturer's instruction.

Fifteen centimeter long branches from one year old 'Arbequina' olive trees (growing in the greenhouse at USDA-ERRC facility in Wyndmoor, PA) were used to induce rooting. The cuttings were first soaked in sterile water for 2 hrs, then immersed for 5 min in 8.2% sodium hypochlorite, and then rinsed three times with sterile water. Leaves were removed from the lower 5 cm portion of the cutting. A fresh oblique cut was made at the bottom of the cutting and the cutting was rolled in hormone powder ('TakeRoot', Garden Safe Brand supplied by Schultz Company, Bridgeton, MO). The hormone-treated portions of the cuttings were gently inserted in 22 × 25 cm plastic pots filled with vermiculite that was soaked with 0.3% hydrogen peroxide made in sterile water. The cuttings were immediately sprayed with sterile water, and then covered with a large beaker to maintain high humidity. The cuttings were sprayed with water 4 times daily and 500 ml of 0.3% hydrogen peroxide were added (hydrogen peroxide provides increased oxygen and suppress microbial growth and hence promote rooting) to each pot twice a week. The pots were kept on heating pads to maintain 25 °C-27 °C, and were given 10 hrs. photoperiod using grow lights. After 7 weeks, the rooted cuttings were divided into two groups; one group was dedicated to be inoculated and other was our uninoculated control

The rooted cuttings were planted in Deepot Cells, 656 ml (Stuewe and Sons, Inc. Corvallis, OR) filled with a mixture of Premier Pro-mix BX and vermiculite (1:1). One group of rooted cuttings was inoculated with 750 spores of *Rhizophagus intraradices* in 1 ml of distilled water. The inoculated and control plants were grown for 3 months in a Conviron growth chamber, set at 10 hrs. photoperiod (daytime temp 25 °C, and night temp 18 °C). After 3 months, the plants were transferred to the greenhouse (in the month of Febraury), where temperature ranged between as low as 10 °C during the night and as high as 27 °C during the day.

R. intraradices fungus were grown in petri dishes on Ri.tDNA carrot roots (St-Arnand et al., 1996).

Nine months after the start of the experiment total number of leaves in each replicate of mycorrhizal and non-mycorrhizal plant were counted and the height of each plant was measured to document differences in plant growth as result of mycorrhizal inoculation.

2.2 Polyphenol Extraction from the Leaves

The leaves sampled from inoculated and control olive saplings were immediately frozen and stored at -80 °C and were ground in liquid nitrogen for extraction. The polyophenols were extracted from powdered leaf material in 80% methanol as described previously (Malik et al., 2015).

2.3 Separation, Quantitation and Identification of Polyphenols in Extracts

The chromatographic separation of the methanol extract was performed as reported before(Malik et al., 2015) with a Nano-Acquity (Waters, Milford, MA) ultrahigh performance liquid chromatographer (UHPLC) equipped with an Acquity UPLC BEH C18, 1.7 µm (1 × 100 mm) column (Waters) maintained at 40 ^{o}C and running at 60µl/minute. The UHPLC-UV chromatogram was obtained by attaching to the UHPLC instrument an Acquity TUV detector (Waters) set to scan at 280 nm. The solvent gradient was modified, starting with water-acetonitrile 90:10 (0.1% formic acid) for 2 minutes and ramped linearly to water-acetonitrile 70:30 (0.1% formic acid) at a

final time of 18 minutes, maintained at that solvent composition for 2 minutes and followed with a columns wash of water-acetonitrile 15:85 (0.1%) formic acid) and returning to the initial condition at 25 minutes. A 10 minutes stabilization time was allowed between injections. Samples for the mycorrhizal and non-mycorrhizal plants were separately combined and mixed with 10 µl of a kaempferol solution (internal standard, 5 µg/ml). Three injections of 4 µl were made for each sample for determination of the concentration change according to the peak height determined by MassLynx v.4.1 software (Waters). The same chromatographic conditions were used for the mass spectrometry analysis.

The mass spectrometry analysis was accomplished by connecting the effluent of the UHPLC instrument to a Synapt G1 quadrupole-time of flight mass spectrometer (Waters) operating in the V mode (resolving power of 8,000 FWHM) and with an electrospray ionization (ESI) probe operated in the positive or negative mode and controlled by MassLynx v.4.1 software (Waters). The instrument parameters were 2.7 kV capillary voltage, 48 V extractor voltage, 300 L/h desolvation gas (N_2) flow, and 120 °C and 150 °C source and desolvation temperatures, respectively. The MS/MS of the deprotonated precursor ions $[M-H]^-$ were obtained by collision-induced dissociation with argon gas at 0.9 ml/min with the collision energy ramped between 6 to 30 eV.

3. Results and Discussion

3.1 Effects of Mycorrhizal Fungi Inoculation on Plant Growth

Olive saplings inoculated with *R. intraradices* at early rooting stage showed increased plant growth relative to the uninoculated controls (Figure 1).

Figure 1. Growth differences in mycorrhizal (inoculated with Rhizophagus intraradices; on the right) and uninoculated (on the left) ‘Arbequina’ olive saplings nine months after rooting and inoculation

The picture was taken nine months after inoculation. The increase in plant height was 146% and the number of leaves were 117% more in mycorrhizal plants compared to uninoculated controls (Table 1). This increased plant growth in mycorrhizal plants is consistent with previous reports that showed increase in growth in different plants, including olives (Artursson et al., 2006; Auge, 2004; Dag et al., 2009; Farzaneh et al., 2011). The new and important findings of this study, however, are changes in polyphenol levels (Table 2).

Table 1. Differences in plant growth between mycorrhizal and uninoculated plants

Treatment	Plant Height (centimeters)	Number of Leaves	Root Colonization
Non Myc	19.6 ± 2.3	50.6 ± 10.8	0%
Myc	48.3 ± 7.6	109 ± 9.0	80.04% ± 2.3%
Percent increase over Non Myc	146%	117%	

Note. All values are Average ± SEM.

All Data Significant with a P value of less than 0.006 using t test for the significance.

Myc = Mycorrhizal plant.

Table 2. Percent change in the levels of polyphenol in the leaves of olive saplings in mycorrhizal plants compared to uninoculated control

Identification of Polyphenols	Peak number	Retention time	Percent increase over unioculated controls	Significantly different at P value using t test
Luteolin-7'-O-Glucoside	1	10.02	107.25%	0.0279
Verbascoside	2	10.27	-100%*	-
Luteolin-4'-O-Glucoside	3	11.47	42.65%	0.0327
Oleuropein	4	13.74	42.44%	< 0.0001
Oleuroside	5	14.46	67.89%	< 0.0001
Oleuropien derivative	6	14.97	32.02%	< 0.0001
Ligstroside	7	16.05	48.21%	0.0166

Note. * Not detected in mycorrhizal plants.

3.2 Effects of Mycorrhizal Fungi Inoculation on Changes in Levels of Polyphenols

Figure 2 shows an HPLC profile identifying various polyphenols in the olive leaf extract, which are similar to our previous reports (Malik & Bradford, 2006; Selin et al., 2012), but with the addition of a few more compounds due to the use of mass spectrometry.

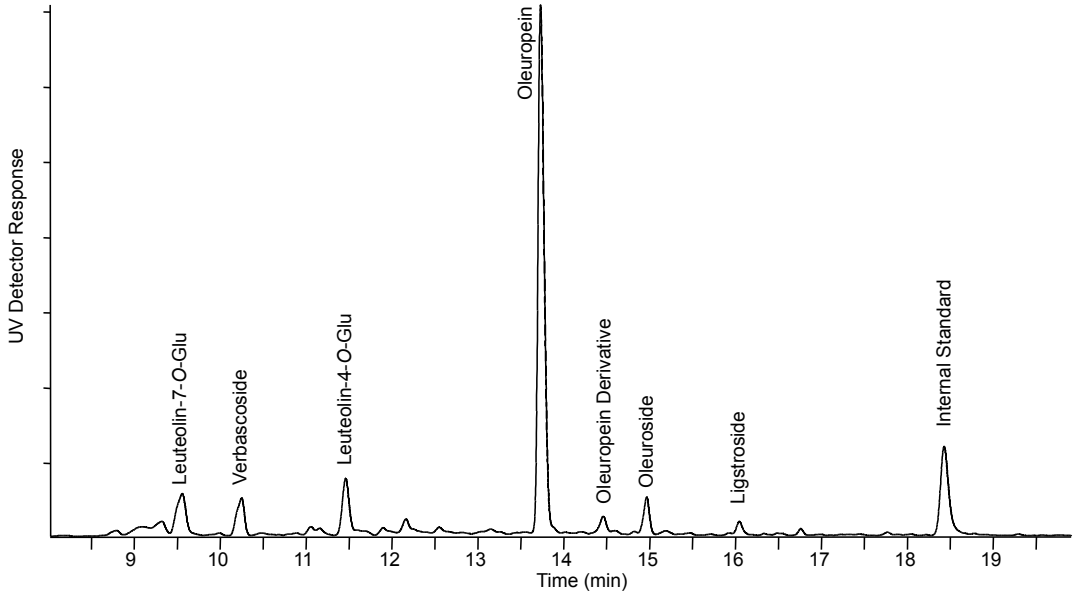

Figure 2. A representative profile of different polyphenols from olive leaf extract separated by HPLC

The identity of the compounds eluting at the corresponding retention time, in Table 2, were determined by using commercially available compounds, comparing retention time and the mass obtained with the mass spectrometer

for the fallowing entrees: leuteolin-7-O-glucose ([M-H]- = 447.04; calculated 447.09, MS/MS fragments at m/z 285), verbascoside ([M-H]- = 621.14, calculated 621.18, MS/MS fragments at m/z 461, and 161), leuteolin-4'-O-glucose ([M-H]- = 447.04; calculated 447.09, MS/MS fragments at m/z 285), and oleuropein ([M-H]- = 539.15; calculated 539.18, MS/MS fragments at m/z 377, 307, 275, and 225) (Savarese et al., 2007). The peaks eluting at 14.46 and 14.97 min have the same mass spectra as oleuropein, with the same fragments at m/z 377; 307; 275; and 225 suggesting two isomers of oleuropein. Savarese et al. (2007) reported oleuroside as an isomer eluting after oleuropein, with the same mass spectrum and accordingly, we are assigning the peak at 14.46 as oleuroside based on its longer retention time in the reversed phase column as was reported before (Savarese et al., 2007; Savournin et al., 2001). No further identification was possible for the peak closer to oleuropein at 14.97 min and is reported as a derivative of oleuropein. The peak eluting at 16.05 was identified as ligstroside, ([M-H]- = 523.15; calculated 523.18, MS/MS fragments at m/z 361, 291, 259 and 101) as reported before (Savarese et al., 2007).

Although the majority of polyphenols identified in the leaves had increased levels in mycorrhizal plants compared to the uninoculated controls, the level of verbascoside decreased to a point where they were below detection (Table 2). This was the only polyphenol species whose levels decreased in mycorrhizal olive saplings, oleuropein which is a major component of olive leaf polyphenols increased by 42%, and its derivative oleuroside and ligstroside increased by 68% and 48%, respectively (Table 2). The greatest increase was found in the levels of luteolin-7'-O-glucoside (107% increase), while its sister compound luteolin-4'-O-glucoside increased by 43% (Table 2). This pattern of increases in certain polyphenol species with decreases in the levels of other polyphenol species, in mycorrhizal plants, compared to controls, has been observed in other plant species (Malik et al., 2015, 2016).

In general, our results show that inoculation of olive sapling's freshly formed roots with mycorrhizal fungi (*Rhizophagus intraradices*) produces beneficial effects on the growth of the plants and improves the levels of its major polyphenols. Olive polyphenols are well known for several health benefits, described before, and therefore, the current study is important as it provides information regarding improving the quality of commercial olive products.

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